



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/DK97/00145 <b>(22) International Filing Date:</b> 3 April 1997 (03.04.97) <b>(30) Priority Data:</b> 0391/96 3 April 1996 (03.04.96) <b>DK</b> <b>(71) Applicant (for all designated States except US):</b> NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK). <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> SØRENSEN, Niels, Henrik [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). <b>(74) Common Representative:</b> NOVO NORDISK A/S; Novo Allé, DK-2880 Bagsværd (DK).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> AN ENZYME FOR DYEING KERATINOUS FIBRES  <b>(57) Abstract</b>  The present invention relates to a dyeing composition comprising an oxidation enzyme derived from the genus <i>Pyricularia</i> , a method for dyeing hair, and the use of a <i>Pyricularia</i> laccase for dyeing keratinous fibres.		

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**Title:** An enzyme for dyeing keratinous fibres

#### **FIELD OF THE INVENTION**

The present invention relates to a dyeing composition for keratinous fibres, such as hair, a method for dyeing keratinous fibres and the use of an oxidation enzyme derived from *Pyricularia* for dyeing keratinous fibres, such as human or animal hair.

#### **BACKGROUND OF THE INVENTION**

It has been used for many years to dye the hair of humans to cover appearing grey hair. The need to do so arises from the fact that grey hair is the first sign of having past adolescence, which can be hard to accept for many people.

Further, during the last few decades hair dyeing has become more and more popular in the western world. At first Punk Rockers and other society critical groups dyed their hair in extreme colours as a part of their protest against the established society, but today especially many young people also use hair dyes (in more soft tints than the Punk Rockers) as a sort of "cosmetic" to change or freshen up their "looks".

#### Hair dyes

In general hair dyeing compositions on the market today can be divided into three main groups:

- temporary hair dyes,
- semi-permanent hair dyes, and
- permanent oxidative hair dyes.

The temporary hair dyes are only intended to change the natural hair colour for a short period of time and usually function by depositing dyes on the surface of the hair. Such hair dyes are easy to remove with normal shampooing.

When using semi-permanent hair dyes the colour of the dyed hair can survive for five or more shampoos. This is achieved by using dyes which have a high affinity for hair keratin and which are capable of penetrating into the interior

of the hair shaft.

Permanent hair dyes are very durable to sunlight, shampooing and other hair treatments and need only to be refreshed once a month as new hair grows out. With such dyeing systems the dyes are created directly in and on the hair. Small aromatic colourless dye precursors (e.g. p-phenylenediamine and o-aminophenol) penetrate deep into the hair, where said dye precursors are oxidized by an oxidizing agent into coloured polymeric compounds. These coloured compounds are larger than the dye precursors and cannot be washed out of the hair.

By including compounds referred to as modifiers (or couplers) in the hair dyeing composition a number of hair colour tints can be obtained. Cathecol and Resorcinol are examples of such modifiers.

Traditionally  $H_2O_2$  is used as the oxidizing agent (colour builder). As  $H_2O_2$  is also a bleaching agent dyeing compositions comprising  $H_2O_2$  are often referred to as "lightening dyes".

The use of  $H_2O_2$  in dye compositions has some disadvantages, as  $H_2O_2$  damages the hair. Further, oxidative dyeing usually demands high pH (normally around pH 9-10), which inflicts damage on the hair and irritate the scalp. Consequently, when using dye compositions comprising  $H_2O_2$ , it is recommendable not to dye the hair often.

To overcome the disadvantages of using  $H_2O_2$  it has been suggested to use oxidation enzymes to replace  $H_2O_2$ .

US patent no. 3,251,742 (Revlon) describes a method for dyeing human hair by dye formation *in situ* (i.e. on the hair). An oxidative enzyme is used to the colour formation reactions at a substantially neutral pH (pH 7-8.5).

Laccases, tyrosinases, polyphenolases and catacolases are mentioned as the suitable oxidation enzymes.

EP patent no. 504.005 (Perma S.A.) concerns a composition for dyeing hair which do not require the presence of  $H_2O_2$  (hydrogen peroxide). Said composition comprises an enzyme capable of catalyzing the formation of polymeric dyes, and

also dye precursors, such as bases and couplers, in a buffer solution. The pH in said composition lies between 6.5 and 8 and said enzyme has an optimal activity in the pH range between 6.5 and 8.

*Rhizoctonia praticola* laccase and *Rhus vernicifera* laccase have a pH-optimum between 6.5 and 8 and can be used to form the polymeric dyes according to this patent.

WO 95/33836 (Novo Nordisk A/S) describes the use of a laccase derived from *Myceliophthora thermophila* which may be used for dyeing of hair.

WO 96/00290 (Novo Nordisk A/S) discloses the use of a laccase derived from *Polyporus pinsitus* for oxidative dyeing of hair.

It is known that *Pyricularia oryzae* laccase may be used oxidation of phenolic azo dyes (see Muralikrishna et al., (1995), Appl. Environ. Microbiol., 61, (12), pp. 4374-4377).

The use of *Pyricularia* laccase for dyeing keratinous materials such as hair is not mentioned and anticipated by said document.

#### **SUMMARY OF THE INVENTION**

The object of the present invention is to provide a permanent dyeing composition for keratinous fibres, such as hair, which has an improved colour development (i.e. dyeing effect).

The terms "colour development" and "dyeing effect" are used interchangeably in the following defining a colour change (measured as DE) of the dyed keratinous fibre in question.

It has surprisingly been found that it is possible to provide such an improved hair dyeing composition by using a laccase derived from a strain of the filamentous fungus genus *Pyricularia* as the oxidation enzyme.

Firstly, the invention relates to a permanent dyeing composition for keratinous fibres, in particular hair, comprising an oxidation enzyme comprising

1) one or more oxidation enzymes derived from a strain of the genus *Pyricularia*,

- 2) one or more dye precursors, and optionally
- 3) one or more modifiers.

In a preferred embodiment of the invention the oxidation enzyme is a laccase derived from a strain of the genus *Pyricularia*, in particular from a strain of the species *Pyricularia oryzae*.

Secondly, it is the object of the invention to provide a method for dyeing keratinous fibres, in particular hair, comprising contacting an oxidative enzyme, such as a laccase, derived from a strain of the genus *Pyricularia*, in the presence or absence of at least one modifier, with at least one dye precursor, for a period of time, and under conditions sufficient to permit oxidation of the dye precursor.

Finally the invention relates to the use of an oxidation enzyme derived from a strain of the genus *Pyricularia* for oxidative dyeing of keratinous fibres, in particular hair.

#### **BRIEF DESCRIPTION OF THE DRAWING**

Figure 1 shows the dose-response (i.e.  $\Delta E$  vs. LACU/ml) for *Pyricularia* laccase and *Polyporus* laccase

#### **DETAILED DESCRIPTION OF THE INVENTION**

The object of the present invention is to provide a permanent dyeing composition for keratinous fibres, such as hair, having improved dyeing effect.

It has surprisingly be found that it is possible to provide such an improved dyeing composition for keratinous fibres by using an oxidation enzyme derived from a strain of the filamentous fungus genus *Pyricularia*.

When using a fixed activity of laccase derived from a strain of the genus *Pyricularia* the colour developed is improved when compared to the same activity of laccase derived from *Polyporus pinsitus* described in WO 96/00290 (Novo Nordisk A/S) (See Example 1).

Further, as shown in Example 2 the dose-response dyeing

effect for *Pyricularia* laccase is higher than for *Polyporus* laccase.

Improved colour development is, in the context of the present invention, defined as a DE higher than the DE value of the above mentioned *Polyporus pinsitus* laccase.

Consequently, in the first aspect the present invention relates to a permanent dye composition for keratinous fibres, such as hair, comprising

- 1) one or more oxidation enzymes derived from a strain of the genus *Pyricularia*,
- 2) one or more dye precursors, and
- optionally 3) one or more modifiers.

In an embodiment of the invention the oxidation enzyme is a laccase derived from a strain of genus *Pyricularia*, such as a strain of *Pyricularia oryzae* e.g. the laccase which can be purchased from SIGMA under the trade name SIGMA no. L5510.

In addition, laccases derived from the genus *Pyricularia* also encompass alternative forms of laccases which may be found in *Pyricularia* as well as laccases which may be found in other fungi which are synonyms or fall within the definition of the genus *Pyricularia*.

It is to be understood that the *Pyricularia* laccases used for dyeing keratinous fibres according to the present invention may be produced homologously or heterologously using especially filamentous fungi, yeasts or bacteria as host cells.

Examples of filamentous fungus host cells include strains of the species of *Trichoderma*, preferably a strain of *Trichoderma harzianum* or *Trichoderma reesei*, or a species of *Aspergillus*, most preferably *Aspergillus oryzae* or *Aspergillus niger*, or yeast cells, such as e.g. a strain of *Saccharomyces*, in particular *Saccharomyces cerevisiae*, *Saccharomyces kluyveri* or *Saccharomyces uvarum*, a strain of *Schizosaccharomyces* sp., such as *Schizosaccharomyces pombe*, a strain of *Hansenula* sp.,

*Pichia* sp., *Yarrowia* sp., such as *Yarrowia lipolytica*, or *Kluyveromyces* sp., such as *Kluyveromyces lactis*, or a bacteria, such as gram-positive bacteria such as strains of *Bacillus*, such as strains of *B. subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. coagulans*, *B. circulans*, *B. lautus*, *B. megaterium* or *B. thuringiensis*, or strains of *Streptomyces*, such as *S. lividans* or *S. murinus*, or gram-negative bacteria such as *Escherichia coli*.

Laccases (benzenediol:oxygen oxidoreductases) (E.C. class 1.10.3.2 according to Enzyme Nomenclature (1992) Academic Press, Inc) are multi-copper containing enzymes that catalyze the oxidation of phenols. Laccase-mediated oxidations result in the production of aryloxy-radical intermediates from suitable phenolic substrates; the ultimate coupling of the intermediates so produced provides a combination of dimeric, oligomeric, and polymeric reaction products. Certain reaction products can be used to form dyes suitable for dyeing keratinous fibres such as hair (see below).

In an embodiment of the invention the *Pyricularia* laccase has improved colour development properties at neutral pH. In the context of the present invention this means that the colour development is improved when using the *Pyricularia* laccase in a dyeing composition having a pH in the range from between 5.0 and 9.0, in particular between 6.0 and 8.0, especially around pH 7.0.

To obtain a suitable dyeing of the keratinous fibres, such as hair, the dyeing composition of the invention must also comprise a dye precursor which is converted into a dye by the oxidation agent which according to the invention is an oxidation enzyme, especially a laccase, derived from a strain of the genus *Pyricularia*, such as a strain of species *Pyricularia oryzae*, especially the above mentioned laccase which can be purchased from SIGMA.



The dye precursor is preferably an aromatic compound e.g. belonging to one of three major chemical families: the diamines, aminophenols (or aminonaphtols) and the phenols.

Examples of such suitable dye precursors include compounds from the group comprising comprising p-phenylene-diamine (PPD), p-toluylene-diamine (PTD), chloro-p-phenylenediamine, p-aminophenol, o-aminophenol, 3,4-diaminotoluene, 2-methyl-1,4-diaminobenzene, 4-methyl-o-phenylenediamine, 2-methoxy-p-phenylenediamine, 2-chloro-1,4-diamino-benzene, 4-amino diphenylamine, 1-amino-4- $\beta$ -methoxyethylamino-benzene, 1-amino-4-bis-( $\beta$ -hydroxyethyl)-amonibenzene, 1-3-diamino-benzene, 2-methyl-1,3-diamino-benzene, 2,4-diaminotoluene, 2,6-diaminopyridine, 1-hydroxy-2-amino-benzene, 1-hydroxy-3-amino-benzene, 1-methyl-2-hydroxy-4-amino-benzene, 1-methyl-2-hydroxy-4- $\beta$ -hydroxyethylamino-benzene, 1-hydroxy-4-amino-benzene, 1-hydroxy-4-methylamino-benzene, 1-methoxy-2,4-diamino-benzene, 1-ethoxy-2,3-diamino-benzene, 1- $\beta$ -hydroxyethyloxy-2,4-diamino-benzene, phenazines, such as 4,7-phenazinedicarboxylic acid, 2,7-phenazinedicarboxylic acid, 2-phenazinecarboxylic acid, 2,7-diaminophenazine, 2,8-diaminophenazine, 2,7-diamino-3,8-dimethoxyphenazine, 2,7-diamino-3-methoxyphenazine, 2,7-diamino 3-methoxyphenazine, 3-dimethyl 2,8-phenazinediamine, 2,2'-[(8-amino-7-methyl-2-phenazinyl)imino]bis-ethanol, 2,2'-[(8-amino-7-methoxy-2-phenazinyl)imino]bis-ethanol, 2,2'-[(8-amino-7-chloro-2-phenazinyl)imino]bis-ethanol, 2-[(8-amino-7-methyl-2-phenazinyl)amino]-ethanol, 2,2'-[(8-amino-2-phenazinyl)imino]-bis-ethanol, 3-amino-7-(dimethylamino)-2,8-dimethyl-5-phenyl-chloride, 9-(diethylamino)- benzo[a]phenazine-1,5-diol, N-[8-(diethylamino)-2-phenazinyl]-methanesulfonamide, N-(8-methoxy-2-phenazinyl)-methanesulfonamide, N,N,N',N'-tetramethyl-2,7-phenazinediamine, 3,7-dimethyl-2-phenazinamine, p-amino benzoic acids, such as p-amino benzoic acid ethyl, p-amino benzoic acid glycerid, p-amino benzoic acid isobutyl, p-dimethylamino benzoic acid amil, p-dimethylamino benzoic acid octyl, p-diethoxy amino benzoic amil, p-dipropoxy amino

benzoic acid ethyl, acetylsalicylic acid, isatin derivatives, such as 2,3-diamino benzoic acid.

In an embodiment of the invention the oxidative enzyme derived from *Pyricularia* is used with the dye precursor directly to oxidize it into a coloured compound.

It is to be understood that dye precursors can be used alone or in combination with other dye precursors. However, it is believed that at least one of the intermediate in the copolymerization must be an ortho- or para-diamine or aminophenol, such as p-phenylenediamine, o-aminophenol, p-methylaminophenol, p-aminophenol, p-toluylenediamine and N-phenyl-p-phenylenediamine. Contemplated are also all dye precursors listed in US patent no. 3,251,742 (Revlon), the contents of which are incorporated herein by reference.

Optionally the hair dyeing composition of the invention also comprises a modifier (coupler) by which a number of hair colour tints can be obtained. In general modifiers are used, as the hair colours resulting from hair dyeing compositions without modifier(s) are usually unacceptable to most people.

Modifiers are typically m-diamines, m-aminophenols, or polyphenols. The modifier (coupler) reacts with the dye precursor(s) in the presence of the oxidative enzyme, converting it into a coloured compound.

Examples of modifiers (couplers) include comprising m-phenylene-diamine, 2,4-diaminoanisole, 1-hydroxynaphthalene( $\alpha$ -naphthol), 1,4-dihydroxybenzene(hydroquinone), 1,5-dihydroxynaphthalene, 1,2-dihydroxybenzene(pyrocatechol), 1,3-dihydroxybenzene (resorcinol), 1,3-dihydroxy-2-methylbenzene, 1,3-dihydroxy-4-chlorobenzene (4-chlororesorcinol), 1,2,3, trihydroxybenzene, 1,2,4-trihydroxybenzene, 1,2,4-trihydroxy-5-methylbenzene, 1,2,4-trihydroxytoluene.

In the second aspect the invention relates to a method for dyeing hair, comprising contacting an oxidation enzyme, such as a laccase, derived from a strain of the genus *Pyricularia*, in the presence or absence of at least one modifier, with at least one dye precursor, for a period of time, and under

conditions sufficient to permit oxidation of the dye precursor.

The dyeing method can be conducted with one or more dye precursors, either alone or in combination with one or more modifiers. Amounts of components are in accordance with usual commercial amounts for similar components, and proportions of components may be varied accordingly.

When using an oxidation enzyme derived from *Pyricularia*, such as the *Pyricularia oryzae* laccase mentioned above, the method for dyeing hair of the invention may be carried out at room temperature and at a pH in the range from 5.0 to 9.0, preferably 6.0 to 8.0, especially around pH 7.

Suitable dye precursors and optionally modifiers are described above.

The use of an oxidative enzyme derived from *Pyricularia*, such as a laccase, is an improvement over the more traditional use of  $H_2O_2$ , in that the latter can damage the hair, and its use usually requires a high pH, which is also damaging to the hair. In contrast, the reaction with an enzyme can be conducted at acidic or neutral pH (below pH 9.0), and the oxygen needed for oxidation comes from the air, rather than via harsh chemical oxidation.

The result provided by the use of the oxidation enzyme derived from *Pyricularia*, such as a laccase, is comparable to that achieved with use of  $H_2O_2$ , not only in colour development, but also in wash stability and light fastness. An additional commercial advantage is that a single container package can be made containing both the laccase and the precursor, in an oxygen free atmosphere, which arrangement is not possible with the use of  $H_2O_2$ .

Also when comparing the colour development using an oxidation enzyme derived from the genus *Pyricularia*, such as a laccase, with a laccase such as the *Polyporus* laccase described above the *Pyricularia* oxidation enzyme gives improved colour development.

## MATERIALS AND METHODS

### Materials:

#### Hair:

6" De Meo Virgin Natural White Hair (De Meo Brothers Inc. US)

#### Enzymes:

Laccase from *Pyricularia oryzae* purchased from SIGMA under the product name SIGMA no. L-5510, lot 54H3398, 389 UNITS/mg solid corresponding to 18.5 LACU/g.

Laccase from *Polyporus pinsitus* described in WO 96/00290, (103 LACU/ml).

#### Dye precursors:

0.1 % w/w p-phenylene-diamine (ppd) in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % w/w o-aminophenol in 0.1 M K-phosphate buffer, pH 7.0.

#### Modifier:

0.1 % w/w m-phenylenediamine in 0.1 M K-phosphate buffer, pH 7.0.

#### Equipment:

Minolta CR200 Chroma Meter for colour measurement

#### Determination of Laccase Activity (LACU)

Laccase activity is determined from the oxidation of syringaldazin under aerobic conditions. The violet colour produced is photometered at 530 nm. The analytical conditions are 19 mM syringaldazin, 23.2 mM acetate buffer, pH 5.5, 30°C, 1 minute reaction time.

1 laccase unit (LACU) is the amount of enzyme that catalyses the conversion of 1.0 mmole syringaldazin per minute at these conditions.

### Assessment of the hair colour

The quantitative colour of the hair tresses are determined on a Minolta CR200 Chroma Meter by the use the parameters  $L^*$  ("0"=black and "100"=white),  $a^*$  ("-60"=green and "+60"=red) and  $b^*$  ("-60" blue and "+60" yellow).

$DL^*$ ,  $Da^*$  and  $Db^*$  are the delta values of  $L^*$ ,  $a^*$  and  $b^*$  respectively compared to  $L^*$ ,  $a^*$  and  $b^*$  of untreated hair (e.g.  $DL^* = L^*_{\text{sample}} - L^*_{\text{untreated hair}}$ ).

$DE^*$  is calculated as  $DE^* = \sqrt{DL^{*2} + Da^{*2} + Db^{*2}}$  and is an expression for the total quantitative colour change (i.e. colour development or dyeing effect).

### **EXAMPLES**

#### **Example 1**

##### Dyeing effect

The dyeing effect of a *Pyricularia oryzae* laccase was tested using the dye precursor p-phenylenediamine and compared with an equivalent activity of *Polyporus pinsitus* laccase under the same reaction conditions.

##### Hair dyeing

1 gram De Meo white hair tresses were used.

4 ml dye precursor solution was mixed with 1 ml laccase on a Whirley mixer, applied to the hair tresses and incubated at 30°C for 60 minutes. The activity of both the *Pyricularia oryzae* laccase and the *Polyporus pinsitus* laccase were 0.048 LACU/ml reaction mixture (pH 7).

The hair tresses were then rinsed with running water, washed with shampoo, rinsed with running water, combed, and air dried.

The  $a^*$ ,  $b^*$  and  $L^*$  was determined on the Chroma Meter and the  $DE^*$  values were then calculated.

A hair tress sample treated without enzyme was used as a blind.

The result of the hair dyeing test is shown in the Table 1.

Table 1

	L*	DL	a*	Da*	b*	Db*	DE
Untreated hair	73.4	-	2.4	-	23.7	-	-
blind (without enzyme)	66.7	-6.7	4.2	1.8	23.2	-0.5	7.0
<i>Polyporus</i> laccase	65.4	-8.0	3.8	1.5	22.6	-1.1	8.2
<i>Pyricularia</i> laccase	37.8	-35.6	3.4	1.0	1.8	-21.9	41.8

As can be seen from Table 1 the colour development (i.e. DE) is improved when using the *Pyricularia oryzae* laccase for dyeing hair in comparison to a corresponding tests using the *Polyporus pinsitus* laccase.

## Example 2

### Dose-response dyeing test of *Pyricularia oryzae* laccase

The dyeing effect of from 0 to 1 LACU/ml *Pyricularia oryzae* laccase was compared with corresponding doses of *Polyporus pinsitus* laccase under the same conditions. 0.1% w/w o-aminophenol (dye precursor) and 0.1% w/w m-phenylene-diamine (modifier) was used.

### Hair dyeing

1 gram white De Meo hair tresses were used.

4 ml dye precursor solution (i.e. 2 ml dye precursor and 2 ml modifier) was mixed with 1 ml laccase in different concentrations (resulting in activities in the reaction mixtures from 0 to 1 LACU/ml) on a Whirley mixer, applied to the hair tresses in a glass beaker and incubated at 30°C under shaking for 30 minutes.

The hair tresses were then rinsed with running water, washed with shampoo, rinsed with water, combed, and air dried.

$a^*$ ,  $b^*$  and  $L^*$  were measured on the Chroma Meter and  $\Delta E^*$  was then calculated.

Hair tress samples treated without enzyme were used as blinds.

The result of the test is displayed in Figure 1. From Figure 1 it can be seen that the *Pyricularia* laccase gives a higher  $\Delta E$  value than the *Polyporus* laccase at equivalent LACU/ml reaction mixture.

**PATENT CLAIMS**

1. A permanent dyeing composition for keratinous fibres, such as hair, comprising an oxidation enzyme characterized in that the composition comprises:

- 1) one or more oxidation enzymes derived from a strain of the genus *Pyricularia*,
- 2) one or more dye precursors, and
- optionally 3) one or more modifiers.

2. The permanent dyeing composition according to claim 1, wherein the oxidation enzyme is a laccase derived from a strain of the genus *Pyricularia*, in particular *Pyricularia oryzae*, especially *Pyricularia oryzae* sold under the product number L-5510.

3. The permanent dyeing composition according to claims 1 and 2, having a pH in the range from 5.0 to 9.0, preferably 6.0 to 8.0, especially about 7.

4. The permanent dyeing composition according to any of claims 1 to 3, comprising a dye precursor selected from the group comprising p-phenylene-diamine (PPD), p-toluylene-diamine (PTD), chloro-p-phenylenediamine, p-aminophenol, o-aminophenol, 3,4-diaminotoluene, 2-methyl-1,4-diaminobenzene, 4-methyl-o-phenylenediamine, 2-methoxy-p-phenylenediamine, 2-chloro-1,4-diamino-benzene, 4-amino diphenylamine, 1-amino-4- $\beta$ -methoxyethylamino-benzene, 1-amino-4-bis-( $\beta$ -hydroxyethyl)-amonibenzene, 1-3-diamino-benzene, 2-methyl-1,3-diamino-benzene, 2,4-diaminotoluene, 2,6-diaminopyridine, 1-hydroxy-2-amino-benzene, 1-hydroxy-3-amino-benzene, 1-methyl-2-hydroxy-4-amino-benzene, 1-methyl-2-hydroxy-4- $\beta$ -hydroxyethylamino-benzene, 1-hydroxy-4-amino-benzene, 1-hydroxy-4-methylamino-benzene, 1-methoxy-2,4-diamino-benzene, 1-ethoxy-2,3-diamino-benzene, 1- $\beta$ -hydroxyethyloxy-2,4-diamino-benzene, phenazines, such as 4,7-phenazinedicarboxylic acid, 2,7-



phenazinedicarboxylic acid, 2-phenazinecarboxylic acid, 2,7-diaminophenazine, 2,8-diaminophenazine, 2,7-diamino-3,8-dimethoxyphenazine, 2,7-diamino-3-methoxyphenazine, 2,7-diamino 3-methoxyphenazine, 3-dimethyl 2,8-phenazinediamine, 2,2'-[(8-amino-7-methyl-2-phenazinyl)imino]bis-ethanol, 2,2'-[(8-amino-7-methoxy-2-phenazinyl)imino]bis-ethanol, 2,2'-[(8-amino-7-chloro-2-phenazinyl)imino]bis-ethanol, 2-[(8-amino-7-methyl-2-phenazinyl)amino]-ethanol, 2,2'-[(8-amino-2-phenazinyl)imino]bis-ethanol, 3-amino-7-(dimethylamino)-2,8-dimethyl-5-phenyl-chloride, 9-(diethylamino)- benzo[a]phenazine-1,5-diol, N-[8-(diethylamino)-2-phenazinyl]-methanesulfonamide, N-(8-methoxy-2-phenazinyl)-methanesulfonamide, N,N,N',N'-tetramethyl-2,7-phenazinediamine, 3,7-dimethyl-2-phenazinamine, p-amino benzoic acids, such as p-amino benzoic acid ethyl, p-amino benzoic acid glycerid, p-amino benzoic acid isobutyl, p-dimethylamino benzoic acid amil, p-dimethylamino benzoic acid octyl, p-diethoxy amino benzoic amil, p-dipropoxy amino benzoic acid ethyl, acetylsalicylic acid, isatin derivatives, such as 2,3-diamino benzoic acid.

5. The permanent hair dyeing composition according to claims 3 and 4, comprising a dye modifier selected from the group comprising m-phenylene-diamine, 2,4-diaminoanisole, 1-hydroxynaphthalene ( $\alpha$ -naphthol), 1,4-dihydroxybenzene (hydroquinone), 1,5-dihydroxynaphthalene, 1,2-dihydroxybenzene (pyrocatechol), 1,3-dihydroxybenzene (resorcinol), 1,3-dihydroxy-2-methylbenzene, 1,3-dihydroxy-4-chlorobenzene (4-chlororesorcinol), 1,2,3-trihydroxybenzene, 1,2,4-trihydroxybenzene, 1,2,4-trihydroxy-5-methylbenzene, 1,2,4-trihydroxytoluene.

6. A method for dyeing keratinous fibres, such as hair, comprising contacting an oxidation enzyme, such as a laccase, derived from a strain of the genus *Pyricularia*, in the presence or absence of at least one modifier, with at least one dye precursor, for a period of time, and under conditions

sufficient to permit oxidation of the dye precursor used for oxidizing the dye.

7. The method according to claim 6, wherein the oxidation enzyme is a laccase derived from a strain of the genus *Pyricularia*, in particular *Pyricularia oryzae*, especially *Pyricularia oryzae* sold under the product number L-5510.

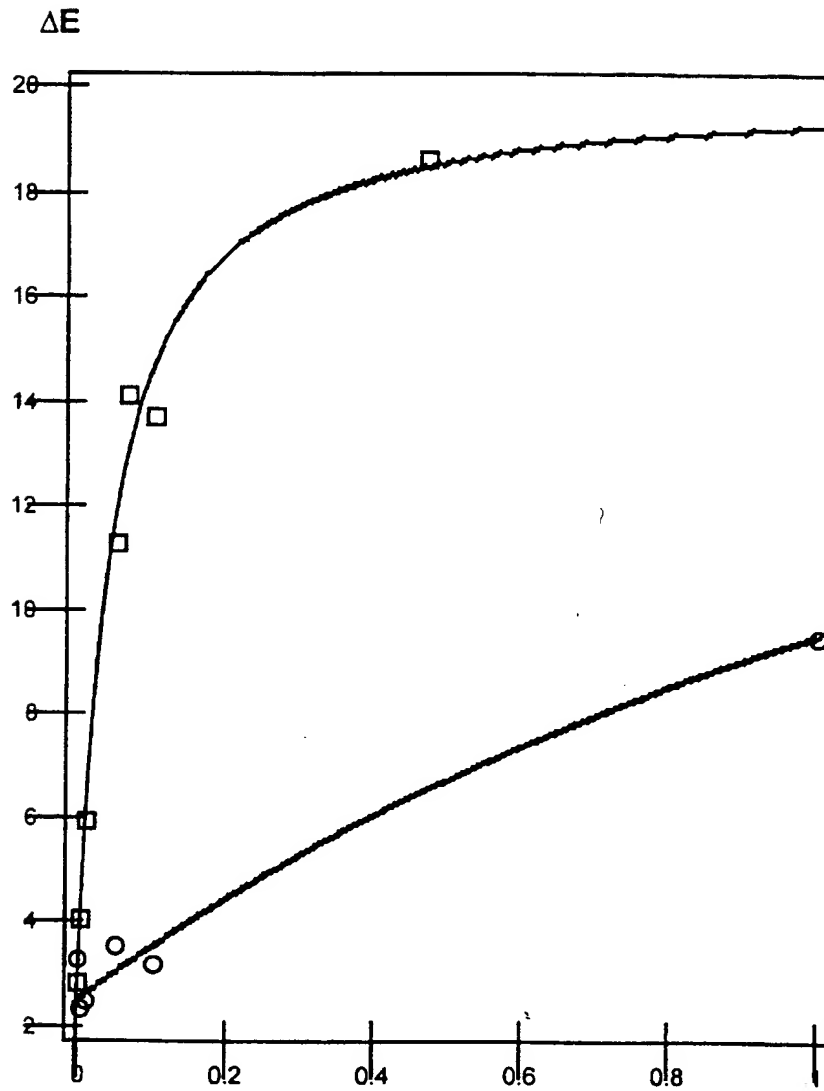
8. The method according to claim 7, wherein the dyeing is carried out at a pH in the range from 5.0 to 9.0, preferably 6.0 to 8.0, especially at about pH 7.0.

9. The method according to claims 6 to 8, wherein the oxidation enzyme is reacted with a dye precursor of claim 4.

10. The method according to claims 8 and 9, wherein the oxidation enzyme is reacted with a dye modifier of claim 5

11. Use of an oxidation enzyme derived from a strain of the genus *Pyricularia* for oxidative dyeing of keratinous fibres, such as hair.

12. The use according to claim 14, wherein the oxidation enzyme is a laccase derived from a strain of the species *Pyricularia oryzae*, especially *Pyricularia oryzae* sold under the product number L-5510.



*Polyporus pinsitus* laccase: O

*Pyricularia oryzae* laccase: □

Figure 1

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 7/13, C09B 67/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, C09B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLUS, WPI

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Applied and Environmental Microbiology, Volume 61, No 12, December 1995, Muralikrishna Chivukula et al, "Phenolic Azo Dye Oxidation by Laccase from Pyricularia oryzae" page 4374 - page 4377 --	1-12
A	WO 9533836 A1 (NOVO NORDISK BIOTECH, INC.), 14 December 1995 (14.12.95), page 16, line 12 - page 17, line 27; page 34, line 20 - page 36, claims 31-42 --	1-12
A	US 3251742 A (SAUL SOLOWAY), 17 May 1966 (17.05.66) --	1-12

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

## \* Special categories of cited documents:

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Date of the actual completion of the international search

25 June 1997

Date of mailing of the international search report

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00145

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9600290 A1 (NOVO NORDISK BIOTECH, INC.), 4 January 1996 (04.01.96), page 48, line 25 - page 54, line 24, claims 37-48  -- -----	1-12

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Information on patent family members

03/06/97

International application No.

PCT/DK 97/00145

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